41. Notes on Preparation of Leiotonin

By Setsuro EBASHI, M. J. A., and Hideko NAKASONE

Department of Pharmacology, Faculty of Medicine,
University of Tokyo, Bunkyo-ku, Tokyo 113

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The method for preparing leiotonin described in previous papers (Ebashi *et al.*, 1975; Mikawa *et al.*, 1977; Mikawa *et al.*, 1978) has become far from being satisfactory from nowadays' points of view. So it is an urgent problem to develop a new method which ensures a high yield of purer preparation of leiotonin.

The method described below is still at a preliminary stage, but may provide us with a starting material for the above purpose.

Materials and methods. One hundred grams of chicken gizzard, minced by a meat grinder, were thoroughly mixed with 400 ml of 0.05 M KCl containing 1 mM NaHCO_3 . After 2 hrs, the suspension was centrifuged (the supernatant contained a lot of myosin, being the starting material for myosin preparation) and to the resulting supernatant was added 400 ml of 0.08 M KCl containing 0.5 mM ATP and 1 mM NaHCO_3 . After 2 hrs the mixture was centrifuged, and 300 ml of 1 mM NaHCO_3 were added to the resulting residue and thoroughly mixed. After 2 hrs the mixture was centrifuged (the supernatant contained a lot of tropomyosin, being used for tropomyosin preparation) and the resulting residue was washed three times with 300 ml of 0.02 M KCl containing 1 mM NaHCO_3 .

The residue (a part of the residue was treated by acetone and the resulting acetone powder was used as actin source) was mixed well with equal volume of 1.2 M KCl and after 5 hrs the mixture was centrifuged at $12,000 \times g$ for 20 min. To the residue an equal volume of 0.6 M KCl was added and the above process was repeated. The resulting two supernatants were combined and dialyzed against 0.05 M KCl containing 1 mM NaHCO_3 . The resulting precipitate (this can be used as the source of natural actomyosin or myosin B) was removed by centrifugation and the supernatant was dialyzed against 0.02 M MgCl_2 . After removing the precipitate by centrifugation, the supernatant was then subject to inverse ammonium sulfate fractionation as follows.

To 100 ml of the supernatant, 53.6 g of finely powdered ammonium sulfate was added and dissolved quickly. The resulting precipitate was centrifuged at $22,000 \times g$ for 10 min. The residue was

Table I. Biological activities of fractions obtained by inverse ammonium sulfate fractionation

Fractions	Leiotonin activity (a)	Kinase activity (b)	a/b	Protein
Original	(100)	(100)	(1)	(100)
Sup I	3	13	0.2	21
Sup II	20	5.7	3.5	33
Sup III	14	0.6	23	10
Sup IV	8	0.5	16	21
	45	20		85

The activity to induce the superprecipitation of gizzard actomyosin in the following conditions was considered to represent the leiotonin activity: 0.02 M KCl, 8mM MgCl₂, 0.02 M Tris-maleate (pH 6.8), 0.4 mM Ca²⁺-GEDTA (glycol-etherdiaminetetraacetic acid, EGTA) buffer (1.3×10^{-5} M Ca²⁺), 0.5mM ATP, 0.16 mg/ml of gizzard actomyosin (myosin : actin=1:1), 20 μ g/ml of gizzard tropomyosin and 0.5 μ g/ml of pig brain calmodulin. The amount of each fraction to induce the same degree of superprecipitation as induced by a specified amount of the original extract was determined and then the total activity of each fraction was calculated. Attention was focused on the profiles of first two minutes of superprecipitation after addition of ATP. The light chain kinase activity was determined by urea gel electrophoretic method of Pierrie and Perry (1970) under the conditions: 2mg/ml myosin, 0.06 M KCl, 8mM MgCl₂, 0.02 M Tris maleate (pH 6.8) and 5 μ g/ml calmodulin (total volume, 0.1 ml) at 25°C for 5 min.

mixed well with two volumes of 1.9 M ammonium sulfate and centrifuged at $22,000 \times g$ for 20 min. The supernatant, designated 'Sup I' in Table I, was subject to the examination of biological activities. The residue was successively extracted with equal volume of 1.5 M and 1.1 M ammonium sulfate and then 1 mM NaHCO₃ in the same way as above, and the resulting supernatants, denoted as 'Sup II', 'Sup III' and 'Sup IV', respectively (Table I), were analyzed for their biological activities.

The procedures for determining the leiotonin and the myosin light chain activities are described in the legend to Table I.

Preparation of other proteins, assay procedures and materials were the same as described in previous papers (Ebashi *et al.*, 1975; Ebashi, 1977; Mikawa *et al.*, 1977).

Results and discussion. In Table I, both the leiotonin and myosin light chain kinase activities of each fraction are presented. Sup III and Sup IV showed high ratios of the leiotonin activity to the kinase activity, but were still heavily contaminated by other proteins. Sup IV cannot be used for further purification because of its low specific activity.

Leiotonin as well as the kinase tends to lose its activity by various

treatments, e.g., by ammonium sulfate fractionation, DEAE-cellulose chromatography and so on. The yield of leiotonin by inverse am-

Fig. 1. Effect of N-ethylmaleimide (NEM) on the leiotonin and kinase activities. The preparation used in this experiment was the fraction of ammonium sulfate between 35 and 55 g per 100 ml of the first extract of gizzard mince (see Materials and methods), which contained a relatively high kinase activity. ●, ▲: Leiotonin activity or superprecipitation-inducing activity. ○: Kinase activity. NEM-treatment was carried out at 0°C for 1 hr in the presence of 1 mM GEDTA (●, ○) or 1.3×10^{-5} M Ca^{2+} (▲) and the reaction was terminated by adding excess dithiothreitol. Other experimental conditions for the leiotonin and kinase activities were essentially the same as those in the legend to Table I.

Fig. 2. The time course of deterioration of the leiotonin and kinase activities. A preparation corresponding to Sup III, preserved at -70°C for 7 months, was thawed and then kept at 0°C . ●: Leiotonin activity. ○: Kinase activity. No change in activities was found due to freezing. The relative activities were determined taking the ratio of the leiotonin activity to the kinase activity (a/b in Table I) in the original extract (corresponding to 'Original' in Table I) as unity. In the case of unfrozen preparation, the rate of fall is usually less than the above.

monium sulfate fractionation is more than 40% of the original extract, which is much higher than that by the ordinary ammonium sulfate fractionation, giving rise to about 25% to 30% in its yield. Furthermore, the preparation made by inverse ammonium sulfate fractionation is more stable. The rate of loss of its activity is less than 25% a week at 0°C and there will be virtually no change if kept frozen, whereas the preparation by ordinary ammonium sulfate fractionation is very labile, its activity being lost abruptly after a week or so.

Generally speaking, the leiotonin activity is more stable than the kinase activity. For example, N-ethylmaleimide affected the kinase activity more intensely than the leiotonin activity (Fig. 1). Utilizing the labile nature of the kinase, deteriorating rapidly with the lapse of time, we could rather easily obtain a preparation which showed a considerable leiotonin activity with virtually no kinase activity (Fig. 2).

Fig. 3. Superprecipitation of gizzard actomyosin without phosphorylation of myosin light chain. Superprecipitation and phosphorylation experiments were carried out in the same conditions as shown in the legend to Table I. The crude leiotonin preparation corresponding to 'Original' in Table I was, after concentration, subject to Ultrogel (AC 44) column equilibrated with 0.5 M KCl including 1 mM NaHCO₃, and early eluate (from 0.05 to 0.1 volume per total bed) was examined; for superprecipitation 6 µg/ml and 3 µg/ml of the eluate were used in the absence and presence of calmodulin, respectively, and for phosphorylation, 60 µg/ml and 30 µg/ml. The arrowhead indicates the position of phosphorylated light chain. Note that in the absence of calmodulin, a marked superprecipitation took place without phosphorylation. The dependence of crude leiotonin on calmodulin became more marked in later eluates, indicating the degree of detachment of leiotonin C from leiotonin A increased in later eluates.

The separation of kinase activity from leiotonin activity in the absence of calmodulin can be made by various procedures, but the most reliable method is to use Ultrogel (AC 44). The fraction thus obtained does not show any kinase activity in the absence of calmodulin, exhibiting a marked leiotonin activity (Fig. 3). This may indicate that calmodulin is easily detached from parent molecule during gel filtration, but leiotonin C is not.

Conclusion. A method for preparing a leiotonin fraction of a high activity with a low light chain kinase activity was described. Reference was also made to a leiotonin preparation which was completely free of the kinase activity in the absence of calmodulin.

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References

- Ebashi, S.: *J. Biochem.*, **79**, 229–231 (1977).
Ebashi, S., Toyo-oka, T., and Nonomura, T.: *ibid.*, **78**, 859–861 (1975).
Mikawa, T., Toyo-oka, T., Nonomura, Y., and Ebashi, S.: *ibid.*, **81**, 273–275 (1977).
Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S., and Kakiuchi, S.: *ibid.*, **84**, 1633–1636 (1978).
Pierrie, W. T., and Perry, S. V.: *Biochem. J.*, **119**, 31–38 (1970).